REMARKS

Claims 5-16, 21-24 and 26-32 were pending. Claims 11-16, 21-24 and 26-32 were withdrawn by the Examiner as being drawn to one or more non-elected inventions. Claims 5-10 are pending. No claim is allowed.

Summary of Examiner Interview

Applicants wish to thank Examiner Lam for the discussion of the Information Disclosure Statement filed June 24 and the pending specification and claims with Laurie Hill, Cary Miller and Tobey Tam on December 12, 2007. In brief, Examiner Lam acknowledged that foreign patent documents and non-patent literature listed on the Information Disclosure Statement filed June 21, 2004 do not need to be submitted if they were previously submitted in a parent application. However, Applicants volunteered to send another copy of any foreign patent documents or non-patent literature to aid the Examiner.

With regards to the objection to the Specification and the Claim rejections under 35 USC § 112, first paragraph cited in the Office Action dated October 31, 2007, the Examiner acknowledges that she interpreted independent Claim 5 to read that the capture antibody has affinity for an analyte as well as an enzyme substrate, which was not an intended embodiment of the present invention and was not intended to be captured within the claims. After reviewing an example of the preferred embodiment of the invention within the Specification, Examiner Lam acknowledged that independent Claim 5 is written grammatically correct and that she misinterpreted it.

Applicants wish to thank Examiner Lam for these acknowledgements and for agreeing to issue another non-final office action on the merits.

Information Disclosure Statement

According to the Examiner, the IDS filed was non-complaint because a legible copy of each foreign patent document and each non-patent literature were not required. As discussed in the interview, these documents were properly submitted in the parent application and thus a second submission is not required. See M.P.E.P. § 609(A)(2). Nonetheless, we provide herewith an additional copy of the non-patent literature documents from IDS for the Examiner's convenience.

The foreign patent documents are not included as the Examiner has already signed the IDS form as having reviewed these documents.

Objections to the Specification

The Examiner object to the specification for allegedly failing ot provide antecedent basis for the claimed subject matter. Applicants traverse this objection.

Applicants respectfully submit that the instant specification provides proper antecedent basis for the claimed subject matter. Applicants wish to point out that the capture zone contains 1) a capture antibody having affinity for the analyte and 2) an enzyme substrate at a test line. Found throughout the specification are details describing the capture zone. For example, in paragraph [0012], the specification states that

> the capture zone containing a capture antibody having affinity for the analyte; and an enzyme substrate at a test line. In one aspect of this preferred embodiment, the substrate is chemically immobilized at the test line. Alternatively, the substrate is immobilized in a mordant under the test line. Still alternatively, the substrate is immobilized in a mordant dispensed within the test line. Preferably, the capture zone further comprises chemical groups incorporated therein, the chemical groups capable of specifically reacting with the product resulting from enzyme action on the substrate.

Another example is found in paragraph [0013], "the capture zone containing an enzyme/mediator for releasing the substrate and a capture antibody at a test line." Furthermore, Figure 2 delineates an example of reagent placement within the lateral flow enzyme immunoassay strip where, "an enzyme/mediator 26 is immobilized in a mordant within or under the test line 28, or attached to the capture antibody 30." See the specification at paragraph [0057].

In view of this disclosure, Applicants submit that the specification provides the necessary basis for the claimed subject matter and request the withdrawal of the objection.

Rejection Under 35 U.S.C. § 112, first paragraph - written description

Claims 5-10 were rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement. The Examiner asserts that " [i]ndependent claim 5, from which claims 6-10 depend, recites in lines 5-6, 'said capture zone containing a capture antibody having affinity for said analyte and an enzyme substrate at a test line.' Howevere,

nowhere else in the specification is a capture antibody having affinity for an analyte and an enzyme substrate disclosed." Applicants traverse this rejection.

Applicants respectfully submit that the specification provides adequate written description for the reasons discussed above.

In view of the above, the withdrawal of this rejection is respectfully requested.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 273102008401. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: December 21, 2007

Respectfully submitted,

By: /Laurie L. Hill/
Laurie L. Hill
Registration No.: 51,804
MORRISON & FOERSTER LLP
12531 High Bluff Drive, Suite 100
San Diego, California 92130-2040
(858) 720-7945

Form PTO-1449

INFORMATION DISCLOSURE CITATION IN AN APPLICATION

(Use several sheets if necessary)

Docket Number 273102008401	Application Number 10/763,466
Applicant: Alan M. NELSON et al.	
Filing Date January 22, 2004	Group Art Unit 1641
Mailing Date June _____, 2004	

U.S. PATENT DOCUMENTS

Examiner Initials	Ref. No.	Date	Document No.	Name	Class	Subclass	Filing Date If Appropriate
	1.	12/1976	3,996,345	Ullman et al.			
	2.	08/1982	4,342,826	Cole			
	3.	10/1984	4,478,944	Gross et al.			
	4.	12/1986	4,632,901	Valkirs et al.			
	5.	05/1987	4,666,830	Wagner			
	6.	10/1987	4,703,017	Campbell et al.			
	7.	02/1988	4,727,019	Valkirs et al.			
	8.	06/1988	4,752,572	Sundberg et al.			
	9.	05/1989	4,828,982	Wagner			
	10.	08/1989	4,857,453	Ullman et al.			
	11.	09/1989	4,868,108	Bahar et al.			
	12.	02/1990	4,904,583	Mapes et al.			
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	25.	11/1997	5,686,315	Pronovost et al.			
	26.	01/1998	5,712,172	Huang et al.			

EXAMINER: DATE CONSIDERED:

EXAMINER: Initial if citation considered, whether or not the citation conforms with MPEP 609. Draw a line through the citation if not in conformance and not considered. Include a copy of this form with next communication to applicant.

Form PTO-1449

INFORMATION DISCLOSURE CITATION IN AN APPLICATION

(Use several sheets if necessary)

Docket Number 273102008401	Application Number 10/763,466
Applicant: Alan M. NELSON et al.	
Filing Date January 22, 2004	Group Art Unit 1641
Mailing Date June _____, 2004	

	27.	02/1998	5,714,389	Charlton et al.			
	28.	03/1998	5,728,587	Kang et al.			
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	36.	05/2000	6,068,859	Curatolo et al.			
	37.	07/2000	6,087,185	Lee-Owen et al.			
	38.	09/2000	6,121,341	Sawhney et al.			

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Examiner Initials	Ref. No.	Date	Document No.	Country	Class	Subclass	Translation YES	Translation NO
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	40.	12/1988	0 296 724	Europe				
	41.	07/1992	WO 92/12428	WIPO				
	42.	01/1994	WO 94/01775	WIPO				
	43.	10/1996	WO 96/34271	WIPO				
	44.	10/1996	WO 96/34287	WIPO				
	45.	02/1997	WO 97/06436	WIPO				

OTHER DOCUMENTS

(including author, title, Date, Pertinent Pages, Etc.)

Examiner Initials	Ref. No.	Title
	46.	Deakin et al., Biochem. J. (1963) 89:296-304
	47.	D'Orazlo et al., Analytical Chemistry (1977) 49(13):2083-2086

EXAMINER:	DATE CONSIDERED:

EXAMINER: Initial if citation considered, whether or not the citation conforms with MPEP 609. Draw a line through the citation if not in conformance and not considered. Include a copy of this form with next communication to applicant.

ACKNOWLEDGMENT

The technical assistance of John Gunnels and Tommy Hardwick in this project is gratefully acknowledged.

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Ion Electrode Measurements of Complement and Antibody Levels Using Marker-Loaded Sheep Red Blood Cell Ghosts

Paul D'Orazio and G. A. Rechnitz*

Department of Chemistry, State University of New York, Buffalo, New York 14214

We introduce a new method, using ion-selective membrane electrodes, for the measurement of immunoagents. The method involves the use of marker loaded, sensitized sheep red blood cell ghosts to produce potential changes in response to varying levels of antibody or complement. The release of marker ion from the cell ghosts, as monitored with the membrane electrode, is shown to be a sensitive measure of the concentrations of antibody or complement under controlled conditions.

Recent advances in the development of bio-selective electrode systems have focused primarily upon the use of immobilized enzymes at electrode surfaces, but it has also been pointed out (*1*) that electrodes suitable for immunomeasurements might be feasible if the selective action of antibody–antigen (Ab-Ag) interactions could be quantitated and coupled to an appropriate indicator electrode. We now report on a new approach to this problem through the use of vesicles, such as sheep red blood cell ghosts (SRBC ghosts), to release an electroactive marker ion in response to the immunoreaction. By using a very large number ($>10^6$) of SRBC ghosts at the electrode, the electrode response can be related to the amount of immunoreactive material present; at the same time, selectivity is achieved through the action of "complement" which functions as a catalyst and amplifier in the lysis of the marker-loaded vesicles. It will be seen that this system, although unfamiliar to most analytical chemists and seemingly complicated in the abstract, is readily capable of yielding analytical measurements of either antibody or complement levels with relatively inexpensive equipment and commercially available components.

Much work, accurately described in several reviews, has dealt with the use of complement in quantitative immunology as well as the resolution and study of the specific actions of the several complement components (*2–5*). The reader is referred to these reviews for information concerning theory of complement action and specific details of complement fixation procedures.

The ability to produce lysis of erythrocytes is the phenomenon which has been exploited to determine the level of complement in biological fluids. The recognition and destruction of a sensitized erythrocyte, one which has bound to it its specific antibody, permits a quantitation, under optimal conditions, of the level of complement or of the anti-erythrocyte species present. In general terms, Figure 1 illustrates the sequence of events that permits a quick screening of test serum for any antibody of interest (Ab_1). Unfixed complement, e.g., that which has not been inactivated by the Ab_1-Ag_1 complex in reaction system 1, is capable of producing lysis of sensitized red blood cells. The extent of oxyhemoglobin release as determined by visual inspection can be related to the presence of the antibody of interest. Alternatively, the system can be made quantitative by assaying the released oxyhemoglobin optically at 541 nm.

Red blood cells are useful as indicators for the determination of complement fixation reagents. However, the use of spectrophotometry in conjunction with red blood cells has the disadvantage that both lysed and unlysed cells must be separated from the reaction mixture before any assay of hemoglobin can be attempted. It would therefore be useful to devise a procedure whereby the release of marker could be measured without any need for separations and with a minimum of sample handling. It may also be desirable to follow the course of the complement fixation reaction by a continuous monitoring of marker release as a function of time. This leads to a consideration of a novel indicator for the complement fixation reaction, e.g., red blood cell ghosts.

It has been known that erythrocytes can be re-formed after osmotic lysis under the proper conditions of osmolarity, temperature and pH, in order to trap any solutes present at the time of re-forming (*6–8*). Cells can be lysed in hypotonic solutions containing low salt concentrations and subsequently re-formed by raising the ionic strength of the lysing medium to isotonicity with the solute or solutes that are to be entrapped in the re-formed cells. Obviously, the limited permeability of blood cells to many electrolytes and nonelectrolytes (*6*) could be quite advantageous for purposes of complement fixation since release of marker could be attributed to complement mediated lysis only.

Humphries and McConnell (*7*) have used mammalian erythrocyte ghosts loaded with the spin label tempocholine chloride to follow complement fixation reactions. The present work describes the use of sheep erythrocyte ghosts loaded with the cation trimethylphenylammonium ($TMPA^+$), which can be conveniently measured using an ion selective membrane electrode.

Figure 2 illustrates the system used in this study. Sheep erythrocyte ghosts containing $TMPA^+$ have binding sites of

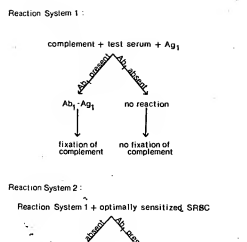

Figure 1. The complement fixation test

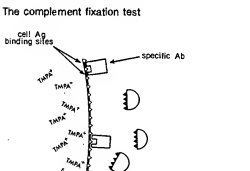

Figure 2. Diagram of complement attack of sensitized sheep erythrocyte ghosts containing $TMPA^+$ marker. Adapted from reference 9

the Forssman antigen available on their surfaces. These are capable of binding with the specific hemolytic antibody. Complement, upon recognizing the complex, lyses the cell through a series of enzymatic reactions. The release of the $TMPA^+$ marker is monitored potentiometrically and related to level of hemolysin antibody or complement, the other component being present in excess.

EXPERIMENTAL

Equipment. All potentiometric measurements were taken on a Corning Model 12 Research pH meter in conjunction with a Beckman Model 1005 10-inch recorder. The trimethylphenylammonium ($TMPA^+$) ion electrode was constructed from an Orion Model 92-19 monovalent cation electrode with a 1×10^{-2} M TMPA chloride internal solution. The ion selective membrane was prepared by the method of Meyerhoff and Rechnitz (*10*). The reference electrode was an Orion Model 90-01 single junction reference electrode and connection between the indicator and reference electrodes was made with a lithiumtrichloroacetate–agar salt bridge.

Potentiometric measurements were made at 37 °C in 15-mL glass thermostated cells with circulation provided by a Haake Model FS water bath/circulator. Any pH adjustments or measurements required were made with a Corning semimicro combination pH electrode. Sonic action was provided by a Bransonic 52 ultrasonic bath. Sheep erythrocyte ghosts were prepared with the aid of a Clay-Adams Sero-fuge.

Reagents. The complement fixation reagents rabbit anti-sheep hemolysin (lyophilized) and guinea pig complement (lyophilized) were obtained from Grand Island Biological Co., Grand Island, N.Y. The guinea pig complement is pooled serum from normal, healthy, adult guinea pigs and was found by the supplier to have titers of no less than 1:130 according to standard methods (*11*). The rabbit antiserum was likewise tested at no less than 1:1500 by these methods. The Sigma Chemical Co., St. Louis, Mo., was the supplier of triethanolamine used for the preparation of triethanolamine buffered saline (TBS) at pH 7.4 as described elsewhere (*12*) and was used as standard diluent for all work described here. Sheep blood cells (stabilized, 10% suspension) were from Baltimore Biological Laboratories.

The following high purity grade reagents were from Fisher Scientific Co.: lithium chloride, calcium chloride, magnesium chloride (heptahydrate), hydrochloric acid, and magnesium sulfate. Trimethylphenylammonium chloride was from Eastman Kodak Co.

Preparation and Evaluation of Sheep Red Blood Cell Ghosts. Sheep red blood cell ghosts were prepared by the method of DeLoach and Ihler (*8*) with some modifications. A 10% suspension of sheep cells was washed three times with equal volumes of TBS by centrifugation of the cells at $1000 \times g$, aspiration of the wash solution, and resuspension in fresh TBS. The final cells were in 50% suspension by decreasing the volume of the final wash to one-fifth the original volume. The cells were then dialyzed for $1^1/_2$ to 2 h vs. cold, 4×10^{-3} M $MgSO_4$ at pH 6.5 to produce osmotic lysis. Re-forming was accomplished by addition of solid TMPA chloride to the lysate, to yield a final concentration of 0.138 M, followed by incubation at 37 °C for 1 h. The re-formed ghosts were then separated from untrapped marker by dialysis overnight vs. a solution of 0.154 M LiCl, 1.5×10^{-4} M $CaCl_2$ and 5.0×10^{-4} M $MgCl_2$ at pH 7.4.

To estimate the total amount of marker trapped, a 200-μL aliquot of ghosts was removed from the dialyzing ghost suspension and diluted to 0.75 mL with TBS. Potentiometric measurement of this sample with the $TMPA^+$ electrode at 37 °C yielded the concentration of untrapped marker. At the same time, a second aliquot was withdrawn and sonicated in an ultrasonic bath for periods up to $1^1/_2$ h at room temperature. Two hundred microliters of the sonicated suspension was diluted with TBS as above and a potentiometric measurement gave an indication of the total marker present, both trapped and untrapped. Similarly, the leakage rate of marker from the SRBC ghosts was estimated by continuous electrode monitoring at 37 °C for periods up to $2^1/_2$ h.

Complement Fixation Procedures Using Sheep Red Blood Cell Ghosts. Lyophilized rabbit anti-sheep hemolysin was reconstituted with distilled water and dialyzed vs. TBS overnight at 4 °C. The antiserum was heated at 56 °C for 30 min to remove any complement activity. Dilutions of the stock solution in the TBS buffer were made in the following concentrations: 1:10000, 1:6000, 1:3000, 1:1500, and 1:750 and frozen until needed. Lyophilized complement was reconstituted prior to use with appropriate amounts of TBS. The following dilutions in TBS were made prior to each complement titration: 1:28, 1:15, 1:10, 1:8, 1:7, 1:6.2, and 1:5.

Hemolysin titrations were done by the following procedure: 0.2 mL of SRBC ghosts were sensitized with 0.2 mL of the various hemolysin dilutions; 10-min incubation at room temperature is considered sufficient for sensitization (*13*). The $TMPA^+$ electrode was then immersed in the cell suspension until a stable potential was reached, usually in 1 min or less. Addition of 5.6 CH_{50} units of complement gave a slight change in potential due to sample dilution. The resulting potential was recorded as E_1. Total sample volume at this point was 0.75 mL. After 15 min, E_2 was recorded; ΔE was taken as $E_2 - E_1$ and plotted as a function of relative hemolysin concentration and stock anti-serum dilution.

Complement titrations were done according to the same procedure except that the cells were sensitized with 0.2 mL of the 1:750 hemolysin dilution. Addition of aliquots of the above mentioned complement dilutions to a final total volume of 0.75 mL represented 0.2, 0.4, 0.55, 0.7, 0.8, 0.9, and 1.1 CH_{50} units respectively. ΔE was plotted as a function of complement units and as a function of stock complement dilution.

A diagram of the experimental setup employed for the hemolysin and complement titrations is shown in Figure 3. Using this arrangement, it was possible to use the $TMPA^+$ ion selective electrode in sample sizes as small as 0.75 mL on a routine basis.

RESULTS AND DISCUSSION

In order to develop a workable method, it is necessary to select a marker material and vesicle so that the combination gives a highly efficient means of trapping marker with minimal

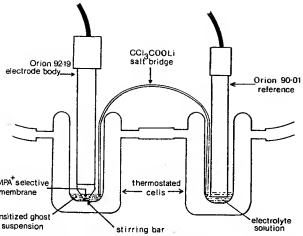

Figure 3. Experimental setup for the determination of complement fixation reagents.

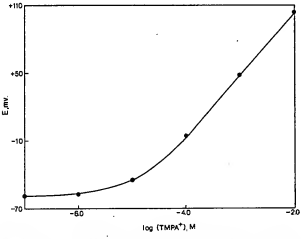

Figure 4. Response curve of the trimethylphenylammonium ion selective electrode in pH 7.4 TBS. Potential measured vs. an Orion single junction reference electrode

eakage until complement mediated lysis effects release.

The use of liposomes or phospholipid vesicles (*14–16*) endered antigenic by the incorporation of lipid antigens into he bilayer was found to be poorly suited for the present work. Specifically, liposomes "loaded" with cations such as Cd^{2+} and Rb^+ as markers exhibited such high rates of leakage that they were useless for this project.

Sheep red blood cell ghosts, in contrast, showed an ability o trap large amounts of $TMPA^+$ marker. Background marker evels could be reduced to as low as 1×10^{-5} M with extensive lialysis. As a result, $TMPA^+$ concentration changes as great s a factor of 10 could be obtained upon complete lysis of the ells. Leakage of marker as a function of time was slow, and egligible in comparison to the trapped marker levels.

Figure 4 shows the response curve of the membrane lectrode to $TMPA^+$ in pH 7.4 TBS buffer. It is clear that he electrode has excellent sensitivity and therefore is useable ver a wide concentration range. The electrode also is highly eproducible from day to day and showed negligible deteriration over an operating period of 1 month. These features re highly attractive for the present work, where sensitivity nd stability of potentiometric response are of prime interest. ince the $TMPA^+$ electrode exhibits a slight Na^+ interference, 'e employed LiCl instead of NaCl for preparation of the TBS uffers to maximize electrode sensitivity. It should be noted, owever, that this substitution is not necessary for routine nalytical work using the proposed procedure.

Figure 5 shows the response of SRBC ghosts, via the release f $TMPA^+$ marker, to varying dilutions of rabbit anti-sheep

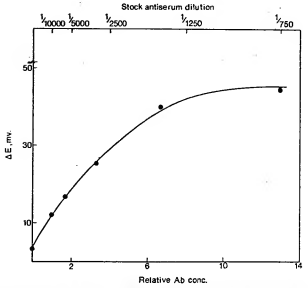

Figure 5. Potentiometric response curve to rabbit hemolytic antibody based on the complement mediated release of $TMPA^+$ marker from SRBC ghosts.

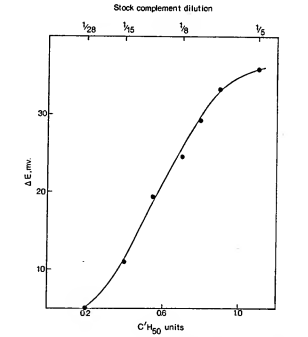

Figure 6. Potentiometric response curve to guinea pig complement based on the release of $TMPA^+$ marker from optimally sensitized SRBC ghosts

hemolysin in the presence of 5.6 $C'H_{50}$ units. The 5.6 $C'H_{50}$ units employed represent a complement excess. As can be seen from Figure 5, the measurements correspond to more than a tenfold change in antibody concentration at the extremely low rabbit antibody sensitivity level indicated on the dilution scale. There is a small release of marker even in the absence of any rabbit antibody. This effect results from the background action of other guinea pig serum components to sensitize the cells. If necessary, this effect could be eliminated by pretreatment of the serum material (*2*); however, such treatment was not needed for the present work.

From Figure 5 it is evident that a 1:750 dilution of rabbit antiserum can be considered an excess of the antibody. Therefore, a 1:750 dilution of antiserum was used to sensitize SRBC ghosts for the purpose of complement titration. Figure 6 shows such a case where optimally sensitized ghosts were lysed to varying degrees by complement. The dual *x*-axis again

shows the good sensitivity of this system for the determination of complement as well as the early saturation effect with increasing CH_{50} units. It is apparent that for purposes of hemolysin titration, 5.6 CH_{50} units represents a safe level of excess complement.

From experimental results shown in Figures 5 and 6, it is clear that workable measurements of antibody and complement levels can be obtained by using ion-selective electrodes in conjunction with SRBC ghosts. The sensitivity of the technique is good, approaching that of the spectrophotometric method. It is conceivable that the sensitivity may be improved by altering the concentration of SRBC ghosts.

The reproducibility of our method is a function of the quality and age of the erythrocyte ghosts used. Specifically, the amounts of marker trapped and untrapped at a given time will determine the range of ΔE values obtained with increasing antibody or complement concentrations. This may vary not only with time of storage of a ghost preparation but also with the reproducibility of preparing the $TMPA^+$ loaded erythrocytes. It is necessary, for best accuracy, to run standard curves immediately prior to unknown antibody or complement determinations.

The use of ion selective electrodes for the quantitation of antibody and complement levels is attractive because electrodes can be employed in turbid biological fluids and, moreover, are capable of giving a means of monitoring such immunoreactions on a continuous basis without sampling or separations. The preparation of the necessary marker loaded SRBC ghosts is extremely simple and the resulting vesicles can be stored until needed.

The experiments described here lay the ground work for further studies on immunoassay using membrane electrodes. By using the complement fixation phenomena as an indicator reaction, it may now be possible to extend the analytical concept to a wide range of antibody determinations with the hope of good sensitivity and easy sample handling.

ACKNOWLEDGMENT

We are grateful for the technical assistance of Eric J. Fogt whose thesis work served to stimulate this research.

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Dynamics of the Desolvating Droplet in a Laminar Flame

Kuang-Pang Li

Department of Chemistry, University of Florida, Gainesville, Florida 32611

Aerosol droplets first undergo desolvation in flames. During desolvation, both mass and volume, i.e., density, of a droplet change continuously. The change in density has a significant effect on the course of flight of the droplet. Taking this into consideration, rigorous expressions are derived for the description of dynamics of the droplet.

In the previous treatment of a flame model (*1*), we indicated that the individual behavior of droplets sprayed into an analytical flame has a profound significance on the spatial distribution of the atomic vapor which, in turn, is closely related to the optical signal observed. Since the atom production in a flame is a very complicated process, several idealized assumptions have to be introduced to make the final expression for the overall process mathematically simple and easy enough to follow. For more rigorous analysis, the dynamics of the droplets at different stages must be described more precisely.

Droplets first undergo desolvation in the flame. The position of the dry aerosol particles from these droplets depends on the velocity and the course of flight of the droplets. Two droplets of the same diameter and from the same origin may end up in different positions when desolvation is complete. This is to say, distribution of the dry salt particles is not only droplet-size dependent but also velocity and course dependent. As a result, the optical signal depends heavily on the dynamics of these desolvating droplets. The purpose of this report is to demonstrate a more precise way to evaluate these dynamic parameters.

DERIVATION

Consider a circular stable laminar flame of uniform composition and temperature T_f (at least uniform in the area of interest). Because of thermal expansion, the flame gases have velocities in both vertical and radial directions. Because of the fact that the flame is stable, we may assume these velocities, denoted as v and v', respectively, to be time-independent.

The origin of the coordinates (r, θ, z) used for describing the dynamics is set at the center of the burner head. A droplet of initial diameter, d_0, in cm, is introduced at (r_0, θ, z_0) with an initial velocity v_0 which makes an angle ϕ with the z-axis. Since no additional θ-directed force is applied to the droplet, the droplet makes no helical movement during the flight. That

2. Each amino acid was incorporated into the protein of the membrane, and the glycerol into the lipid, without giving rise to other residues.

3. The presence of high concentrations of penicillin had no effect on the rates of incorporation.

4. Both novobiocin and streptomycin inhibited the incorporation of amino acids, and, to a much smaller extent, of glycerol; vancomycin inhibited the incorporation of amino acids and glycerol equally. There was no evidence for a selective inhibition, by any of these antibiotics, of incorporation into the membrane fraction compared with the total cell protein and lipid.

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'Glucose 6-Phosphate-Dehydrogenase' Activity and Thiol Content of Thymus Nuclei from Control and X-Irradiated Rats

BY HELEN DEAKIN, MARGERY G. ORD AND L. A. STOCKEN
Department of Biochemistry, University of Oxford

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Creasey & Stocken (1959) showed that the formation of acid-labile phosphate by nuclei isolated from rat thymus gland was completely prevented by exposing the animals to a 100 r. dose of X-rays. The possible function of 'glucose 6-phosphate dehydrogenase' (EC 1.1.1.49) in this nuclear phosphorylation (Ord & Stocken, 1962*a*) led to an examination of the activity of the enzyme after irradiation. The results suggested that alterations in the concentration of thiol (SH) compounds in the nuclei from normal rats might influence the activity of the dehydrogenase and that such alterations might be produced by X-irradiation. Glutathione and glutathione reductase (EC 1.6.4.2) have been described in nuclei from calf thymus (Stern & Timonen, 1954; Wang, 1962), and a study of nuclear thiol groups has been made by Klouwen (1962). The present paper indicates how the method of preparation of the nuclei affects the nature and amount of SH compounds found. The interactions between 'glucose 6-phosphate dehydrogenase' and glutathione reductase have also been examined and shown to complicate the analysis of the effects of X-irradiation on the nuclei.

METHODS

Animals. These were taken from the laboratory strain of Wistar rats. Whenever possible we used females that were put from the time of weaning into a room with a 12 hr. light period, from 8 a.m. to 8 p.m. In all cases the animals were of 100–140 g. body weight (4–6 weeks old).

Nuclei. These were prepared as described by Ord & Stocken (1961) in either 'ionic medium' [KCl (85 mM), NaCl (8·5 mM), $CaCl_2$ (2·5 mM), $MgCl_2$ (2·5 mM) and triethanolamine hydrochloride (5 mM), adjusted to pH 7·2] or in 'sucrose medium' [sucrose (0·25 M), $CaCl_2$ (5 mM) and

(5 mM), at pH 7·2]. In some experiments the nuclei
e prepared in sucrose medium and finally suspended in
e medium. The final concentration of the suspension was
ally the equivalent of one thymus/ml. of medium.

Glucose 6-phosphate dehydrogenase'. 'Glucose 6-phos-
te-dehydrogenase' activity was determined spectro-
tometrically, in the presence of 1 mM-glucose 6-phos-
te and 0·1 mM-$NADP^+$, from the increase in absorption
340 mμ produced by NADPH. A portion (2 ml.) of
ear suspension was added to 2 ml. of medium containing
substrates and centrifuged immediately at 3500*g* for
n. The absorption was measured at 1–2 min. intervals
10–12 min. at room temperature (18°). The absorption
eased linearly for 15–20 min. No attempt was made to
rate glucose 6-phosphate dehydrogenase and 6-phospho-
onate dehydrogenase (EC 1.1.1.44).

Glutathione reductase. This was determined by the de-
se in absorption at 340 mμ in the presence of 0·1 mM-
ized glutathione (GSSG) and 0·1 mM-NADPH. The
edure was as described for 'glucose 6-phosphate
ydrogenase'.

Thiols. These were assayed with either *p*-chloromercuri-
zoate (Boyer, 1954) at pH 7·2 or with di-(2-carboxy-3-
ophenyl) disulphide (distributors: R. N. Emmanuel
, London) (Ellman, 1959). Reduced glutathione (GSH)
tritional Biochemicals Corp., Cleveland, Ohio, U.S.A.)
used as a standard. A portion (0·5 ml.) of nuclear
ension was diluted with either 2·5 ml. of medium and
ml. of 10 mM-di-(2-carboxy-3-nitrophenyl) disulphide
1·5 ml. of medium and 1·0 ml. of 0·3 mM-*p*-chloro-
uribenzoate dissolved in medium. After various times
ncubation at room temperature (18°) the suspensions
centrifuged at 3500*g* for 1 min. and the supernatants
ved for spectrophotometric readings at 412 or 235 mμ
ectively.

Adenosine triphosphate and pyrophosphate. The ATP and
yrophosphate in the nuclear preparations were measured
deproteinization with trichloroacetic acid and removal
he acid with ether. ATP was determined by the
od of Holton (1959) and pyrophosphate by the use of
yrophosphatase (Bailey & Webb, 1944).

Deoxyribonucleic acid. DNA was determined by the di-
ylamine method of Burton (1956).

Arginine-rich and arginine-rich histones. These were iso-
d from nuclei according to the method of Daly & Mirsky
4–55).

Emulsions of linoleic acid peroxide. These were prepared
oxygenation for 20 min. at room temperature (18°) of
eic acid in sucrose medium (cf. Lewis & Wills, 1962).
peroxide content was determined as described by
ner, Clever & Peters (1947).

Radioautography. This was performed as described by
gerald, Simmel, Weinstein & Martin (1953). '[1-^{14}C]-*N*-
rylmaleimide' (0·05 μc; specific activity 8 μc/mg.; from
artz Bio-Research Inc., Mount Vernon, N.Y., U.S.A.)
incubated with 0·3 ml. of nuclear suspension and
ed to 1 ml. with medium. After 0, 15 or 60 min. at
temperature the suspensions were centrifuged and the
ear residues were washed twice with unlabelled *N*-ethyl-
imide (2 ml.; 50 mg./100 ml. of medium). The nuclei
then suspended in 1 ml. of medium, 0·5 ml. was with-
n and plated on to aluminium planchets for determi-
on of ^{14}C activity (Nuclear–Chicago gas-flow counter),
the remainder was used for radioautography. Kodak
AR-10 stripping film was used and 3–4 weeks were allowed for exposure. In experiments with nuclei in sucrose medium the ^{14}C activity in 0·05–0·1 ml. of the final suspension was assayed by scintillation counting (type 6012; Isotope Developments Ltd.).

X-irradiation. Exposures of 200 r. or less *in vivo* were given in the Department of Radiotherapy, the Churchill Hospital, Oxford. The characteristics were: half-value layer, 1·1 mm. Cu; peak voltage, 220 kv at 15 mA. In most of the experiments with 1000 r. *in vivo* the irradiations were performed at the Medical Research Council Radiobiological Research Unit, Harwell. Here the characteristics were: half-value layer, 2·5 mm. Cu; peak voltage, 250 kv at 14 mA. The irradiations *in vitro* were carried out in the University Department of Botany. Characteristics were: unfiltered, 70 kv at 5 mA.

RESULTS

'Glucose 6-phosphate dehydrogenase' in thymus nuclei. The confirmation of the radiosensitivity of nuclear phosphorylation (Ord & Stocken, 1962*b*) prompted an examination of enzyme systems that might be involved in the phosphorylation and that might be affected by X-irradiation. 'Glucose 6-phosphate-dehydrogenase' activity appeared to be diminished in nuclei isolated in ionic medium from thymus glands of rats that had received 88–100 r. 15 min. earlier (Table 1). A dose of 5000 r. *in vitro* did not inhibit the dehydrogenase activity in a rat-liver homogenate (Kunkel, Höhne, Maas & Schubert, 1955), so that direct inactivation of the enzyme itself is unlikely. The nuclear enzyme was not affected by the presence of pancreatic or micrococcal deoxyribonucleases nor by pancreatic ribonuclease (M. G. Ord & L. A. Stocken, unpublished work), so that damage to the enzyme through the release of nucleases (Bacq & Alexander, 1961) was improbable. The range of activity found in both control (Table 1) and irradiated nuclear preparations was, however, disturbingly broad, and a critical examination was therefore undertaken of factors that might alter the activity.

It was observed that, after nuclei had been prepared in ionic medium, the activity of the enzyme was increased by nearly 100% (Table 1) by storing the preparation at 0° for 30 min. before centrifuging. In the experiments with the dehydrogenase obtained from irradiated rats, care was taken that the nuclear suspension was assayed immediately after isolation of nuclei. Centrifuging of a freshly prepared nuclear suspension in a Spinco rotor SW 39 (38000 rev./min. for 15 min.) did not increase the activity in the supernatant, but, when the nuclear sediment obtained after centrifuging for 1 min. at 3500*g* was resuspended and the activity in the resulting (second) supernatant measured, 'glucose 6-phosphate dehydrogenase' was again detectable with an activity of about half that

present in the first supernatant. These results showed that the dehydrogenase was fairly readily extracted from nuclei prepared in ionic medium. The decrease in enzyme activity found after resuspension of the nuclei may explain the decreased phosphorylation found in twice-washed nuclei prepared in ionic medium (Ord & Stocken, 1962*a*).

Since these results suggested that the dehydrogenase activity might be due to absorption of cytoplasmic components, nuclei were prepared in sucrose medium. J. A. Smit & L. A. Stocken (unpublished work) have found a greater loss of protein from nuclei prepared in ionic medium than from nuclei prepared in sucrose medium. Higher activity of 'glucose 6-phosphate dehydrogenase' in sucrose medium (Table 1) supports their finding and, since there was no increase in extractability of the enzyme after standing, it seems that the enzyme is more firmly bound than in nuclei prepared in ionic medium. This was confirmed when nuclei that had been prepared in sucrose medium were subsequently suspended in ionic medium and allowed to stand for 10 min. at room temperature before centrifuging. This gave a greater dehydrogenase activity in the extract than by the other methods. Though these results indicated that great care was required in the preparation of nuclei to obtain reproducible results, further experiments showed that interference in the measurement of the enzyme could arise because of the presence in the supernatants of enzyme systems that used NADPH.

GSSG (0·1–1·0 mM) lowered the apparent activity of the dehydrogenase (Table 2) but this effect was considerably diminished by 10 μM-Zn^{2+} ion, which inhibits glutathione reductase (Fawaz & Fawaz, 1962). The presence of this enzyme in the nuclear extracts was confirmed by direct assay with 0·1 mM-GSSG and NADPH.

Thiol content of nuclei. The adverse effects of GSSG on the measurement of dehydrogenase activity suggested that the effects of X-irradiation might be caused by radiochemical oxidation of GSH present in the nuclei.

Determination of the total thiol content of nuclei prepared in ionic medium at pH 7·2 showed that deproteinized extracts contained only small and irreproducible proportions of the total thiol content. The methods of Boyer (1954) and Ellman (1959) gave the same values for total thiol content on identical samples of nuclei prepared in ionic medium. The reaction was comparatively slow, especially with *p*-chloromercuribenzoate, when the reaction was not complete until 30–45 min. after the addition of the reagent. The addition of 10 μM-Zn^{2+} ion had no effect on the concentration of thiol

Table 1. *'Glucose 6-phosphate-dehydrogenase' activity in nuclei obtained from rat thymus gland*

Five animals were used/nuclear preparation. A portion (2 ml.) of the nuclear suspension was diluted with 2 ml. of incubation mixture and the production of NADPH was followed spectrophotometrically for 10–15 min. The numbers of experiments are given in parentheses. Activity is expressed as μm-moles of NADPH formed/min./mg. of DNA phosphorus at 18°.

	Activity
Nuclei prepared in ionic medium:	
Control (12)	1·35 ± 0·485
Nuclei from rats which received 88–100 r. 15 min. earlier (6)	1·09 ± 0·420
Nuclei sedimented at 3500*g*-min. (1)	1·51
Nuclei sedimented at 10^6*g*-min. (1)	1·51
Nuclei washed once (1)	1·72
Nuclei washed twice (1)	0·88
After standing for 30 min. at 0° (2)	2·58
Nuclei prepared in sucrose medium:	
Control (5)	2·98 ± 0·611
Nuclei extracted at 0° (2)	0·66
After standing 30 min. at 0° (2)	2·98
Nuclei extracted at 18° with ionic medium (2)	10·46

Table 2. *'Glucose 6-phosphate dehydrogenase' and glutathione reductase interactions in nuclei from rat thymus gland*

In the first group of experiments nuclei were prepared in ionic medium, and in the second group they were isolated in sucrose medium and extracted with ionic medium. Five animals were used for each preparation. Enzyme activity is expressed as μmoles of NADPH produced or utilized/min./mg. of DNA phosphorus at 18°.

Substrate and coenzyme	Variant	Enzyme activity (control)	Effect of variant (% of control)
Group 1			
1 mM-Glucose 6-phosphate + 0·1 mM-NADP+	+1 mM-GSSG	1·35	30 (4)
	+ 0·1 mM-GSSG	1·35	68 (4)
	+ 10 μM-GSSG	1·35	100 (1)
Group 2			
1 mM-Glucose 6-phosphate + 0·1 mM-NADP+	+ 10 μM-$ZnSO_4$	10·13	126
	+ 0·1 mM-GSSG	10·13	74
	+ 0·1 mM-GSSG + 10 μM-$ZnSO_4$	10·13	110
1 mM-Glucose 6-phosphate + 0·1 mM-NADP+ 0·1 mM-GSSG + 0·1 mM-NADPH	+ 10 μM-$ZnSO_4$	10·8	117
	+ 10 μM-$ZnSO_4$	1·34	0
	+1 mM-Glucose 6-phosphate	1·34	0

oups, and chromatographic analysis of acid tracts of the nuclei failed to establish the presence reproducible amounts of glutathione.

Isolation of lysine-rich and arginine-rich histones owed that the SH content of nuclei prepared in nic medium was mainly attributable to the ginine-rich histone, and, in agreement with evious work (Daly & Mirsky, 1954–55), no thiol oups were detectable in the lysine-rich fraction. the total SH content 67% was associated with e arginine-rich histone fraction and 83% of the I groups of the histone reacted with di-(2-rboxy-3-nitrophenyl) disulphide in 5 min. The w recovery of SH may be due to the presence of owly reacting thiol compounds that are not tracted from the nuclei by acid treatment.

Nuclei prepared in sucrose medium contained ry much higher amounts of SH groups (Table 3), might be expected from the greater loss of oteins from nuclei isolated in ionic medium. alysis of the supernatant obtained when nuclei epared in sucrose were extracted with ionic edium showed that diffusible thiols were present. romatographic analysis of the acid extract from clei prepared in sucrose medium indicated the esence of glutathione as well as higher-molecular-ight SH compounds. It therefore seems probable at preparations of nuclei in ionic medium did not ow the retention of reproducible amounts of ociated glutathione.

The effects of 10 μM-Zn^{2+} ion on the total SH ntent of nuclei isolated in sucrose medium able 3) and on the 'glucose 6-phosphate-dehydrogenase' activity (Table 2) suggest that these nuclei also contained small amounts of GSSG, whose reduction would normally be catalysed in the extracts by glutathione reductase and NADPH. Nuclei prepared in ionic medium contain all four species of nicotinamide coenzyme (M. G. Ord & L. A. Stocken, unpublished work). The presence of Zn^{2+} ions did not abolish the slowly reacting component in the thiol reaction. Of the SH content of nuclei prepared in sucrose medium 69% was associated with the arginine-rich histone fraction.

In view of the effects of X-irradiation *in vivo* on nuclei isolated in ionic medium, it was interesting that the highest thiol contents were found with nuclei that had been prepared in sucrose and extracted with ionic medium.

Effect of X-irradiation on the thiol content of nuclei. The potential interactions between glutathione reductase and disulphide compounds present or produced in nuclei made it advantageous to investigate the effects of X-irradiation *in vitro* when controls could be directly compared and when glutathione reductase could be inhibited by Zn^{2+} ions. Irradiation with 200 r. *in vitro* had no detectable effect on the thiol content of 10 μM-GSH in 0·02M-phosphate buffer, pH 7·2, but 1000 r. decreased the concentration by 43% (cf. Barron & Flood, 1950). Irradiation of nuclei prepared in ionic medium failed to show any effect on their total SH content, but if nuclei were prepared in sucrose a decrease in SH content was apparent (Table 4). If no Zn^{2+} ions were present the decrease in thiol content was only found at the first spectrophotometric reading (total time from the end of irradiation to measurement, 10 min.), but when 10 μM-Zn^{2+} ion was present the decrease was apparent at both time-intervals and was indeed still found in nuclei that had been kept at 0° for 30–45 min. after irradiation. This suggested that oxidized thiol compounds could be reconverted into the reduced form by the glutathione reductase present in the preparations.

An attempt was made to isolate the thiol compound that might have been oxidized. A dose of 1000 r. was given to 5 ml. of nuclei prepared in sucrose medium. The control and irradiated preparations were treated with 1 ml. of 30% (w/v) trichloroacetic acid, and the acid was removed from the deproteinized supernatants with ether. Only 30% of the total SH content of the nuclei was extracted into the supernatant, and no difference was found between extracts from control and irradiated nuclei. In control preparations of nuclei in sucrose medium about 20% of their total SH groups remained in the supernatant after centrifuging the suspension for 1 min. at 3500*g*, and this fraction also was unaffected when the nuclei were given 1000 r. Thus when nuclei are suspended in

Table 3. *Thiol content of nuclei isolated from rat thymus gland*

Nuclei were incubated at pH 7·2 with di-(2-carboxy-3-rophenyl) disulphide or *p*-chloromercuribenzoate at 18°. er 5–45 min. the preparations were centrifuged at 00*g* for 1 min. and the supernatants used for spectro-otometric assay. The numbers of experiments are given parentheses. Thiol content is expressed as μm-moles of /mg. of DNA phosphorus.

	Thiol content	
	Ellman (1959) method	Boyer (1954) method
clei prepared in ionic medium:		
Total SH	308±38 (15)	280 (5)
SH reacting in 5 min.	255 (7)	84 (2)
clei prepared in sucrose medium:		
Total SH	469±33 (7)	—
SH reacting in 5 min.	375 (5)	—
Effect of $ZnSO_4$		
Control	525, 426	—
+ 10 μM-$ZnSO_4$	482, 399	—
clei prepared in sucrose and spended in ionic medium:		
Total SH	666, 620	—

sucrose medium the radiosensitive thiol components are not readily extractable from the nucleus.

When X-irradiation was given *in vivo* 200 r. produced no significant effect on the thiol content of nuclei isolated in ionic medium, but with 1000 r. increased SH reactivity was found (Table 5). This was confirmed by using ^{14}C-labelled *N*-ethylmaleimide, when the total ^{14}C activity of the washed nuclei increased with the time of incubation with *N*-ethylmaleimide and showed higher counts with nuclei from the irradiated rats. When nuclei were prepared in sucrose medium after X-irradiation *in vivo* two effects were detected. In the absence of Zn^{2+} ions an initial depression in thiol content, found when nuclei were incubated for 5 min. with the reagent, was followed by a slight increase in activity after 30 min. of incubation. This increase was confirmed radioautographically (Table 7), and, when uptake of ^{14}C-labelled *N*-ethylmaleimide was measured, 15 min. and 60 min. of incubation showed 11 and 12% increases respectively in uptake in the irradiated nuclei. It is assumed that during the lengthier manipulations of the nuclei the glutathione reductase reversed the initial lowering of the SH content. When 10 μM-Zn^{2+} ion was present in the sucrose medium throughout the preparation of the nuclei 1000 r. *in vivo* gave a fall in nuclear SH content which was detectable after both 5 min. and 30 min. of incubation with di-(2-carboxy-3-nitrophenyl) disulphide. In this experiment, as with irradiation *in vitro*, there was no decrease in SH content of that fraction (20%) of the thiol activity which was present in the supernatant obtained after centrifuging the nuclear preparation for 1 min. at 3500 *g*.

Radioautography of thiol groups. ^{14}C-labelled *N*-ethylmaleimide was incorporated into an insoluble compound clearly localized in the nucleus.

Table 4. *Effect of X-irradiation* in vitro *on the thiol content of nuclei from rat thymus gland*

Nuclei were prepared in sucrose medium. A portion (5 ml.) of the preparation was irradiated at 0° and the thiol content of the control and irradiated suspensions was measured by using di-(2-carboxy-3-nitrophenyl) disulphide. When present, the final concentration of $ZnSO_4$ was 10 μM. Thiol content is expressed as μm-moles of SH/mg. of DNA phosphorus.

Exposure (r.)	$ZnSO_4$	Time of incubation with SH reagent (min.)	Thiol content	
			Control	Irradiated
1000	–	5	431	382
	–	30	436	481
1000	+	5	392	344
	+	30	531	450
1000	+	5	428	360
	+	30	488	412
1000	–	30	492	497
	+		541	511

Table 5. *Effect of X-irradiation* in vivo *on the thiol content of nuclei isolated from rat thymus gland*

The animals were killed 15–60 min. after irradiation, and nuclei were prepared from control and exposed rats. Six rats were used/group. In some of the experiments in which 200 r. was given the SH groups were measured by using both di-(2-carboxy-3-nitrophenyl) disulphide and *p*-chloromercuribenzoate; in the other experiments only the method of Ellman (1959) was used. The numbers of experiments are given in parentheses. Thiol content is expressed as μm-moles of SH/mg. of DNA phosphorus.

Exposure (r.)	Time after end of exposure (min.)	Time of incubation with SH reagent (min.)	Thiol content	
			Control	Irradiated
Nuclei isolated in ionic medium:				
200 (4)	15	25	314	325
1000 (1)	15	25	299	353
1000 (1)	60	25	270	348
Nuclei isolated in sucrose medium:				
1000 (1)	60	5	289	255
1000 (1)	60	30	357	366
1000 (1)	60	5	363	302
(10 μM-$ZnSO_4$ present)		30	551	411
200 (3)	60	30	446	465
(10 μM-$ZnSO_4$ present)				

e number of nuclei showing the presence of label reased with time (Table 6), and from a differential alysis it appeared that the larger thymocytes ok up the label more rapidly and to a greater ent than in the small thymocytes (Table 7). The rease in SH content of nuclei isolated in ionic dium after exposing the rats to 1000 r. was due inly to an increase in SH content of individual clei rather than to an increase in the number of clei reacting. The action was principally on the all thymocytes, in which the numbers of grains/ cleus appeared to be higher in the irradiated pulation.

Radiomimetic effects of linoleic acid peroxide and lized glutathione. The possibility that lipid oxides are produced in tissues by X-irradiation been considered by Horgan, Philpot, Porter & odyn (1957), who found evidence for peroxide rmation in mice after exposure. Lewis & Wills 62) have shown that linoleic acid peroxide oxidizes SH groups of cysteine very readily, and GSH and papain more slowly.

Linoleic acid peroxide in the concentration used did not affect the absorption at 412 mμ that had been produced by reaction of di-(2-carboxy-3-nitrophenyl) disulphide and 50 μm-moles of glutathione, but when the peroxide was added immediately before the reagent a slight inhibition was found (Table 8), as described by Lewis & Wills (1962). The SH content of the nuclear preparation was lowered by the addition of linoleic acid peroxide; the nuclear SH components were more sensitive to peroxide than was GSH.

Since oxidation of nuclear thiol groups could be produced by linoleic acid peroxide we decided to see if oxidized SH compounds would have any effect on nuclear phosphorylation. GSSG was used as a model and the ability of the nuclear preparations to phosphorylate was examined by measuring the pyrophosphate formation of the nuclei during

Table 6. *Radioautographic analysis of the uptake of* ^{14}*C-labelled N-ethylmaleimide into nuclei isolated in ionic medium from thymus gland of normal and X-irradiated rats*

In Expt. 1, the nuclei were isolated 15 min. after irradiation, and in Expt. 2 60 min. after irradiation. The radiation dose in each experiment was 1000 r.

Expt. no.	Time of incubation with *N*-ethyl-maleimide (min.)	Control: No. of nuclei counted	Control: Percentage with 2 or more grains	Irradiated: No. of nuclei counted	Irradiated: Percentage with 2 or more grains
1	0	719	27·8	529	26·0
	15	481	48·0	421	54·8
	60	256	70·7	372	80·4
2	0	568	28·7	583	37·6
	15	639	39·2	626	41·6
	60	583	56·5	487	53·4

Table 7. *Analysis of grain counts in nuclei from thymus gland of control and X-irradiated rats killed* 15 *or* 60 *min. after exposure to* 1000 *r.*

Nuclei were distinguished as small nuclei and 'others'. The small nuclei comprised about 80% of the total population. Nuclei were isolated in ionic medium, except in Expt. 3, when they were isolated in sucrose medium. Unless otherwise stated, the nuclei were incubated for 60 min. with ^{14}C-labelled *N*-ethylmaleimide. In Expt. 1 the rats were killed 15 min. after exposure; in Expts. 2 and 3 they were killed 60 min. after X-irradiation.

Expt.	Type of nuclei	No. of nuclei counted (and those with more than 2 grains)	Percentage of population with grain counts: 2–4	5–7	8–10	> 10
1	'Others'*	41 (32)	56	25	10	9
	'Others'	37 (32)	19	34	19	28
	Small	152 (91)	72	22	6	0
	Undifferentiated controls	(133)	66	21	8	5
	Undifferentiated 1000 r.	(112)	55	36	4	5
2	Small (controls)	(227)	70	23	6	1
	Small (1000 r.)	(241)	65	31	3	1
3	Small (controls)	(143)	53	31	11	5
	Small (1000 r.)	(168)	40	33	20	7

* Incubated with ^{14}C-labelled *N*-ethylmaleimide for less than 1 min.

incubation at 0°, in the presence and absence of GSSG. Pyrophosphate formation exceeding the disappearance of ATP originally present in the nuclear suspensions has been used as a measure of nuclear phosphorylation. The excess of pyrophosphate arises from the utilization of ATP for activation of amino acids, and possibly of other substrates, for synthetic reactions in the nuclei (Ord & Stocken, 1962*a*). In the presence of 1 mM-GSSG the formation of pyrophosphate was diminished to about 30% of that in the control nuclei (Table 9).

DISCUSSION

Ord & Stocken (1961, 1962*a*) showed that nuclei prepared in ionic medium took part in both phosphate transfer reactions and in the activation of amino acids. From the results reported above it is clear that marked loss of protein from the nucleus occurs in this type of preparation (see also Allfrey, 1959) and this presumably accounts for the variability in glucose 6-phosphate-dehydrogenase concentrations in these nuclei. A more important factor in the dehydrogenase assay was interference from the glutathione-reductase system. This system had been investigated in calf-thymus nuclei by Stern & Timonen (1954) but no measurements were reported of total thiol content of the nuclei. Complete analysis of the thiol groups in these preparations has not yet been achieved but the major contribution that arginine-rich histone(s) make to the total thiol content of nuclei from rat thymus was unexpected. Glutathione appears to account for a comparatively small proportion of the total.

The concentration of glutathione reductase probably accounted for the effects of GSSG on 'glucose 6-phosphate dehydrogenase', but the effects of GSSG on nuclear phosphorylation are less easily interpreted. Nuclear phosphorylation requires the presence of oxygen (Allfrey, Mirsky & Osawa, 1955; W. A. Creasey, unpublished work) so that it seems unlikely that NADPH produced by 'glucose 6-phosphate dehydrogenase' is normally completely reoxidized through the glutathione-reductase system. The inhibitory action of GSSG therefore seems attributable either to diversion of NADPH from oxidation which is usually linked to phosphorylation, or to a disturbance produced by GSSG in the normal thiol–disulphide equilibria in nuclear proteins, whose functions are still unknown. Alterations in thiol–disulphide equilibria of nuclear proteins are thought to occur during cell division (see Mazia, 1961); it is nuclei from the larger thymocytes, showing most of the mitotic activity of the thymus (Sainte-Marie & Leblond, 1958), that have the higher SH reactivity in radioautographs.

Two effects of X-irradiation on nuclear thiol groups have been detected. In nuclei isolated in sucrose medium a decrease in SH content was found after irradiation both *in vivo* and *in vitro*. The fall was transient if glutathione reductase was not inhibited. Glutathione reductase is believed to be specific for GSSG (Knox, 1960), but, since GSH was probably a comparatively minor contributor to nuclear SH groups and since interactions between GSH and protein disulphide groups have been described (see Knox, 1960), initial oxidation of SH groups on proteins cannot be ruled out. The second effect of X-irradiation, most obvious in nuclei prepared in ionic medium, was an increase in thiol groups in nuclei from the smaller thymo-

Table 8. *Effect of linoleic acid peroxide on the thiol content of thymus nuclei prepared in sucrose medium*

0·1 M-Linoleic acid in ethanol was diluted with 9 vol. of sucrose medium. After oxygenation of the emulsion for 20 min. at 18°, it was filtered; 0·3 ml. was diluted with 2·2 ml. of medium, and 0·5 ml. of nuclear suspension was added. Approx. 3 min. later 0·02 ml. of di-(2-carboxy-3-nitrophenyl) disulphide was added. The control assays were performed in the presence of 0·3 ml. of oxygenated sucrose containing 10% (v/v) of ethanol. Thiol content is expressed as μm-moles/3 ml.

Peroxide added (μm-moles/3 ml.)	Thiol content (control)	Decrease in thiol content caused by peroxide
Not known	23·5	6·2
20·4	31·1	8·55
27·5	24·6	10·8
27·5	10·6*	1·4

* In this experiment reduced glutathione was used in place of the nuclear preparation.

Table 9. *Effect of oxidized glutathione on pyrophosphate formation by nuclei isolated in ionic medium*

The reaction was stopped by the addition of trichloroacetic acid. ATP and pyrophosphate were determined after the removal of the acid from the deproteinized extracts. The reaction was stopped at 0 min. or after 30 min. of gentle shaking at 0°. Concentrations are expressed as μm-moles/mg. of DNA phosphorus. P_i, Inorganic phosphate; PP, pyrophosphate.

Initial concn. of phosphate		Change in concn. of phosphate after 30 min.					
		Control			+1 mM-GSSG		
ATP	PP	P_i	ATP	PP	P_i	ATP	PP
5·7	17·9	+35·8	−3·4	+23·1	+58·9	−3·9	+5·3
3·5	29·3	+39·2	−0·8	+21·2	+42·5	−0·8	+8·2

es. This disturbance probably occurred in both
es of preparation, although in nuclei retaining
ger amounts of SH compounds the effect was
sked by the decrease ascribed to oxidation of
tone SH groups. It is not clear whether the two
ions of X-rays are related; the increase in SH
itent was seen in nuclei prepared in ionic medium
h at 15 and at 60 min. after irradiation *in vivo*,
licating that it was a fairly immediate effect. It
s not, however, detected after irradiation *in vitro*
ither type of nuclear preparation. The increased
content might originate from non-histone thiol
ups, which may account for 30% of the total,
t SH reactivity can be increased in control
clei prepared in sucrose if the final suspension is
de in ionic medium. This suggests that the
her ionic strength of ionic medium (I 0·11)
npared with that of the sucrose medium
0·015) might cause partial dissociation of the
cleoprotein structure and permit SH groups that
re unavailable in the sucrose medium to become
ctive. A similar explanation can be offered for
increased SH content after 1000 r. *in vivo*.

These changes in thiol compounds indicate
her profound alterations in nuclear structure
er X-irradiation. Production of SH groups may
the direct result of the ionizing events, but the
idation may be partly consequential on the
mation of lipid peroxides after irradiation
organ *et al.* 1957). A decrease in the total SH
itent of rat thymus after exposure has been
orted by Ashwood-Smith (1961), and Kedrova,
itokol'skaya & Rodionov (1961) have found a
l in extractable SH content of liver nuclei after
00 r. *in vivo*. It is possible that oxidation of
itone SH groups may be associated with the
otection apparently exerted by the protein of
oxyribonucleoprotein against damage to DNA
er X-irradiation *in vivo* and *in vitro* (Peacocke &
eston, 1961). It has already been postulated that
effects of GSSG on nuclear phosphorylation may
through changes in protein thiol–disulphide
uilibria, and such changes could also account for
radiosensitivity of this process and might be
pected to have an effect on the actual mechanism
spindle formation in mitosis and so provide a
ochemical factor in the arrest of this process after
irradiation.

SUMMARY

1. A study has been made of the thiol groups
esent in nuclei from rat thymus gland. Of the
tal thiol content of the nuclei 70% was associated
th arginine-rich histones.

2. Proteins, including those containing thiol
oups, are lost from nuclei isolated in ionic
edium. Nuclei isolated in sucrose medium
ucrose (0·25 M), calcium chloride (5 mM), and tris
(5 mM), at pH 7·2] retain reproducible amounts of thiol compounds and 'glucose 6-phosphate dehydrogenase'.

3. The reactivity of thiol groups in nuclei isolated in sucrose medium is increased if the final suspension is made in ionic medium.

4. '[1-^{14}C]-*N*-Ethylmaleimide' has been used in conjunction with radioautography to investigate the distribution of thiol groups in the nuclear population from rat thymus.

5. X-irradiation *in vivo* or *in vitro* decreases the concentration of thiol groups in nuclei isolated in sucrose medium; this effect is transient if the glutathione reductase, present in the nuclei, is not inhibited by 10 μM-Zn^{2+} ion.

6. X-irradiation *in vivo* increases the number of reactive thiol groups especially in nuclei from small thymocytes; this effect is most obvious in nuclei isolated in ionic medium where the inhibitory action of X-irradiation on nuclear thiol groups is not detected.

7. Linoleic acid peroxide, in concentrations approximately equimolar to the thiol concentration, decreased the thiol content of the nuclei.

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The Formation of Metabolites from Cephalosporin Compounds

BY CYNTHIA H. O'CALLAGHAN AND P. W. MUGGLETON
Glaxo Research Ltd., Stoke Poges, Buckinghamshire, and Greenford, Middlesex

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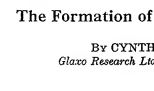
The discovery of cephalosporin and the elucidation of its structure has led to the preparation of a new series of compounds with antibacterial activity. The nucleus of the cephalosporins consists of a dihydrothiazine ring fused to a β-lactam ring (Abraham & Newton, 1961).



Cephalosporin C has an α-aminoadipoyl group in the 7-position (X) and is the only member of this series of compounds that has so far been found in natural products. The cephalosporins differ from the penicillins in that no analogues are produced when precursors such as phenylacetic acid are incorporated in the fermentation medium. The α-aminoadipoyl group can, however, be removed from cephalosporin C by hydrolysis (Loder, Newton & Abraham, 1961) or by treatment with nitrosyl chloride (Morin, Jackson, Flynn & Roeske, 1962), and the 7-amino group can then be substituted if desired. The first cephalosporin analogue to be prepared in this way was 7-phenylacetamidocephalosporanic acid (sodium salt) (called 'phenylacetyl cephalosporin').

In laboratory studies, which are not reported in detail below, we have shown that phenylacetyl cephalosporin has an antibacterial spectrum similar to that of benzylpenicillin, with the added advantages of a greatly increased stability to acid and to penicillinase. Against experimental *Staphylococcus aureus* infections in mice, phenylacetyl cephalosporin gave irregular results in which the protection obtained was not parallel with the dose given. Although it has a low toxicity for mice, some animals receiving low doses were protected, whereas some having larger doses died.

To explain these irregular results, in which no clear-cut end points were obtained, a variable metabolic decomposition of the substance to a compound with decreased antibacterial activity was postulated, and the work reported below describes the investigation of the fate of phenylacetyl cephalosporin and other cephalosporins in the mammalian body.

EXPERIMENTAL

Chromatography and bio-autographs. A suitable solution in 30 μl. quantities was applied to Whatman no. 1 papers, buffered with 0·05M-sodium phosphate at pH 6·0. Undiluted serum and urine samples were applied to the paper in the same volume but, where it was expected that the antibiotic concentration would be low, several (up to five) applications were made on the same spot, the paper being dried in a stream of warm air between applications. The solvents used were: (*a*) butan-1-ol–ethanol–water (4:1:5, by vol.); (*b*) propan-1-ol–water (7:3, v/v). The chromatograms (descending) were allowed to run for 18 hr. After drying, the papers were placed on nutrient–agar plates seeded, for phenylacetyl cephalosporin, with 1% of a 24 hr. broth culture, diluted 1:100, of *S. aureus* (NCTC 7447). For cephalosporin C or *N*-dinitrophenyl cephalosporin C, 1% of a 1:500 dilution of a spore suspension of *Bacillus subtilis* (ATCC 6633) was used. The papers were removed after 15 min., and the plates were incubated at